## Contract N68171-94-C-9101. Final report (14/2 -97)

# 1 Introduction

One of the main aims of the present project (Contract N68171-94-C-9101) was to reach a design for a compact and rugged portable flow cytometer as part of a monitoring system for defense against air and water borne bacteria and other biological particles in biological warfare. The performance of the instrument, in terms of sensitivity, precision and measuring rate would have to be appropriate for accurate detection and characterization of such organisms. In our previous reports we have shown how bacteria can be counted and characterized by their light scattering properties, reflecting size and shape characteristics, their DNA content and their response to various types of antibiotics. Such investigations are essential to determine the performance criteria that should be the basis for the design of an adequate instrument. In one report we outlined the theoretical framework for the performance of flow cytometers as a guide for rational design of such instruments. In this final report we outline a design of an instrument which we believe fill the performance criteria that have been reached during this project.

In view of the relatively short time available for this task our aim has been to base the design entirely on technology that has been proved to work in the context of flow cytometry. Thus, we have consciously shied away from experimenting with new concepts, except where it is necessary to fulfill the special requirements of this instrument, in particular with regard to compactness and ruggedness. No working prototype of this instrument has been constructed as yet. However, all the individual components has been tested in the appropriate context. Hence, we fell very confident that when the system is put together it is going to work according to the specifications.

# 2 Technical background



## 2.1 Light sources

There are two major types of FCMs: those using a laser as the excitation light source and those using an arc lamp. The gas lasers currently employed, mostly argon lasers, are excellent for excitation of dyes with an absorption peak close to the wavelength of the laser. For example a 488 nm Ar laser is perfect for excitation of FITC (fluorescein-isothiocyanate), but suboptimal for dyes with peak absorption at higher wavelengths, such as Texas red, and not usable at all for

dyes requiring shorter excitation wavelengths, such as Dapi, Hoechst bisbenzimidasols and Mithramycin. Further disadvantages of these lasers are a high power consumption, i.e. of the order of 10000 times the output of light, and that they are fairly bulky and not very resistant to mechanical shock and vibration. Laser diodes are compact, rugged and much more energy efficient than gas lasers. However, the vast majority of dyes used in flow cytometry require excitation light of wavelengths below 550 nm, while the most common types of laser diodes are emitting in the red or infrared and can therefore not be used with appropriate fluorescent dyes currently available. However, laser diodes emitting around 534 nm are now available and can be used with certain dyes, notable cyanide derivatives. Although such dyes have a limited range of applications, in particular there is none which is highly specific for DNA, the green laser diode is definitely useful for some purposes.

### 2.2 Laser-based instruments

The central part of any flow cytometer is the optical system. All laser-based flow cytometers have essentially the same optical configuration, that is a configuration with three main axes which are perpendicular to each other and intersecting in the same point, namely the laser beam, the sample flow and the axis of the detection optics for fluorescence and large angle light scattering. A prerequisite for proper function of the instrument is that these three axes meet in a common point, i.e. the laser focus, with a precision of a few micrometers. This presents a problem partly because alignment may be tricky and difficult to maintain, and partly because such a configuration is inherently susceptible to mechanical shock.

### 2.3 Arc lamp-based instruments

Arc lamp-based systems typically employ epi-illumination, which is to say that excitation and fluorescence detection are carried out through the same lens. Hence, two of the main axes of the system coincides and are thereby automatically parfocal and aligned. This greatly facilitates alignment and increases the resistance against mechanical shock and vibration. The lens used for this purpose is usually a microscope objective with high numerical aperture. This implies that the excitation light fills a very wide cone, which eliminates the orientation artifacts seen with laser-based instruments where the excitation light is contained within a very narrow cone.

A major advantage of the arc lamp-based system is that the lamp, which may contain either xenon or mercury or both, emits all across the UV and visible part of the spectrum. Hence, by selecting appropriate filters it can be used for essentially any one of the large number of fluorescent dyes that are being used in flow cytometry. The arc lamp has a much better power efficiency than gas lasers and is more resistant to mechanical shock. Arc lamps are also much cheaper to buy and to operate. However, the long term stability of arc lamps does not match that of the lasers, and their life time is much shorter than for laser tubes, i.e. in the range 200 to 1000 hours, depending on type. Clearly, a combination of a diode laser and an arc lamp could be a solution to this dilemma. Hence, the present design facilitates the use of either light source, either separately or simultaneously. However, at the present time we are not aware of any bacteriological application which requires the latter modality.

## 3 New design

The flow cytometers currently on the market are relatively bulky with volumes upward from about 200 liters and weights from around 60 kg. Thus, they are not very suitable for field work or for operation in mobile units. However, there is ample room for improvement in this respect. Based entirely on technology which is already being used in flow cytometry we have designed an instrument which may be classified as portable. The overall dimensions are about 40x40x40 cm, that is a total volume of 64 liters and a weight, excluding 10 liters of sheath and waste water, around 20 kg. The performance characteristics of this instrument are similar to those of the best commercial products, although it does not facilitate "sorting", that is, on-line physical separation of the cells according to their measured characteristics.

### 3.1 Optics

The optical bench contains 4 PMT fluorescence detectors and 2 PMTs for detection of low and large angle light scattering. The optical configuration is based on the same working principles as that of the Bryte HS (Bio-Rad, Inc. Hercules, CA), although it is packed much more densely. Thus, the entire optics is situated within a volume of about 20x25x12 cm. It employs high grade microscope optics, including a NA = 1.3 oil immersion lens for epi excitation/detection of fluorescence, and a dark field configuration for light scattering detection (1). The optics encompasses filter blocks which are plug-in units that can be replaced in seconds as required when the instrument is switched to new applications.

### 3.2 Light sources

The instrument is designed to be used either with a 100 W arc lamp, which may contain mercury, xenon or both, depending on which are the main applications of the instrument, or a diode laser emitting at 534 nm. The design also facilitates the use of both light sources simultaneously with separate excitation foci and separate fluorescence detection pathways. The excitation intensity is monitored by a photodiode. About 2 % of the excitation light is diverted to this monitor via a semitransparent mirror. The signal from the photodiode can be fed to the signal amplifiers and thereby used to compensate for any fluctuation in the intensity of the excitation light. The photodiode signal can also be read by the software which can thereby keep track of the performance of the light source and give off warnings in case of abnormal behavior.

### 3.3 Fluidics

The detection of bacteria and other microorganisms put great demands on the quality of the sheath water, especially with regard to both chemical pollutants and particulate matter. Reliable supply of such water may become a problem under field conditions. Hence, we have been experimenting with a system of recirculation of the water. The recirculation system comprises a chemically active filter which also removes particles of sizes down to 0,3 μm, that is, to well below the size of even very small bacteria. The system has been found to remove all of the fluorescent dyes most commonly used in flow cytometry to a level where they cause no detectable fluorescence of unstained cells. The filters have sufficient capacity to last for daily use over at least one year. The main advantage of this system is that the total water volume is reduced from about 10 liters in the standard instrument to about 100 ml. Thus, the compactness of the instrument is increased and the risk of water spillage into optics and electronics, which is a potential hazard in a portable unit, is greatly reduced. An additional advantage of this fluidics system is that the instrument becomes fully closed relative to the environment. Most dyes used in flow cytometry are generally quite toxic and quite a few of them mutagenic. In the recirculation system the dyes end up in the chemical filter which can eventually be disposed of in a safe manner, e.g. by incineration.

### 3.4 Electronics

The electronics is essentially that of the Bryte HS which has been proved to yield histograms with negligible noise and distortion. It provides both linear and logarithmic amplification of the signals from all six detectors, which are all photomultiplier tubes. In addition to the analog electronic the system encompasses a microprocessor-based controller board which governs all electronic functions, as well as an ADC board which contains a separate ADC converter for each detector channel and FIFO buffers to maintain an even flow of data to the computer. The computer is a standard PC with a Windows operating system. A lap top computer may be used. Alternatively the computer may be integrated in the instrument.

### 3.5 Software

The software is that of the Bryte HS including a few modifications. In addition to acquisition and storage of data in both histogram mode or list mode this software includes all the standard functions of high grade flow cytometry, including nested gating, automatic histogram scaling, histogram statistics, peak channel detection, DNA histogram analysis, etc.

An important feature of the software is that it can be set up for different user levels, from the highest level where the user has access to every function of the system and can set up the whole system to his own specifications, to the lowest level where the user can only activate and deactivate the system and initiate data acquisition, while a given set of measuring data is automatically displayed and/or presented on hardcopy. Automatic sequences of sampling, data acquisition, data analysis and display may be set up according to the specifications of the user.

## 4 Conclusion

It is fully feasible to construct a flow cytometer which is sufficiently compact and lightweight to be portable and with adequate performance specifications for detection, enumeration and characterization of bacteria and other microorganisms. The entire design is based on well proven principles, technology and technical solutions that have been tested in flow cytometry with satisfactory results. As noted above, this instrument has not yet been realized as a working unit, although the various components have been proven in practical tests. The time required to build up a working prototype and to develop a production model will depend on the resources available for the task. I believe that given adequate funding (somewhere in the range US $ 50 - 100 000) it should be possible to build a working prototype in about 3 months. Development of

a production model would have to be in collaboration with Bio-Rad, which holds the licenses to several patents used in the design. Hence, the time and resources required for that stage of the project can only be settled with their participation.